Romanian Journal for Plant Protection, Vol. X, 2017
ISSN 2248 – 129X; ISSN-L 2248 – 129X

INSECTS' SUSCEPTIBILITY TO THE ACTIVE SUBSTANCE OF A BIOINSECTICIDE FOR COLORADO BEETLE CONTROL

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Abstract: Mass rearing of test insects in the laboratory requires standardized techniques and facilities. The mass rearing of Colorado beetle (*Leptinotarsa decemlineata*) under controlled laboratory conditions is expensive and laborious. This paper presents preliminary studies for testing a complex entomopathogenic bioproduct with simultaneous ingestion and contact action on other test insects that can be mass reared outside the growing season of solanaceous crops. The active substance of the complex bioproduct consists in contagious propagules with contact action (*Beauveria bassiana*) and with ingestion action (*Bacillus thuringiensis* var. *tenebrionis* and *Fusarium oxysporum*). Contagious propagules with contact action were represented by spores of *B. bassiana* native isolates, selected on the criterion of biological compatibility with selective pesticides and belonging to RDIPP's entomopathogenic microorganisms collection, recognized by the international authority NCAIM, Hungary. Contagious propagules with ingestion action were represented by spores of a commercial *B. thuringiensis* strain and a native strain of *F. oxysporum*. These strains were used for individual submerged cultivation. The pathogenic mixture was prepared by mixing the microbial suspensions (bacterial and fungal) in a ratio of 1/1/1, v/v/v. In order to ensure both contact and access of insects to contaminated food, the pathogenic mixture has been included into the whole food following a diet bioassay procedure. The pathogenic inoculum doses /1g of feeding substrate were 2.35×10^8 (*B. bassiana*), 3.76×10^2 (*B. thuringiensis*) and 22.5×10^7 (*F. oxysporum*). Insects belonging to Ord. Lepidoptera (*Plodia interpunctella*, *Galleria mellonella*), respectively Ord. Coleoptera (*Tenebrio molitor*) were characterized in terms of susceptibility to the active substance of bioproduct (*B. bassiana - B. thuringiensis – F. oxysporum* microbian mixture). Test insects exhibited different degrees of susceptibility to the pathogen mixture, recording mortality of 91% (*P. interpunctella*), 66% (*T. molitor*) and 33% (*G. mellonella*). The susceptibility assessment was based on the larval mortality rates, recorded over 48 hours for 10 days.

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Key words: biological control, Colorado beetle, B.bassiana, B. thuringiensis, F.oxysporum

INTRODUCTION

Successful use of entomopathogenic bioinsecticides is determined by the existence of a quality control method that certify the reliable supply of a stable, viable and virulent product with a constant concentration and purity. Specialized laboratories in the world have specific bioassay procedures for biological quality control, according to the objective pursued: control of contaminants, control of active ingredients (spores, conidia), control of humidity of biomass, control of viability, virulence maintenance and testing, determination of the host range. Mass rearing of test insects on artificial diets offers the most reliable method of obtaining large and continuous supplies of insects for insecticide testing, biological control etc.

Potato (*Solanum tuberosum* L.) is an important crop worldwide and the amount of potato yield is influenced especially by the degree of damage caused by Colorado potato beetle (CPB) *Leptinotarsa decemlineata* Say, (Coleoptera: Chrysomelidae), the most important defoliating pest (Igrc et al., 1999); this insect needs permanent control measures for preventing economic damage (Kalushkov & Batchvarova, 2005). The most commonly used

method of CPB management, the application of insecticides, has resulted in the rapid development of resistance to most of the active substances (Scott et al., 2003). Due to the development of insecticide resistance the biological control is a major challenge. Entomopathogenic microorganisms are known as a sustainable alternative for potato protection; *Beauveria bassiana* and *Bacillus thuringiensis* var. *tenebrionis* are important natural enemies of the CPB, with a long history of development as larvicides for potato foliage protection (Andrei, 2002).

B. thuringiensis is a widespread, spore-forming soil bacterium. Some Bt strains produce proteinaceous crystals (δ-endotoxins) during sporulation that are highly toxic to insects. For *B. thuringiensis*, the entry route into its insect host is through the oral cavity, during feeding. Following ingestion by the larval stage of insects, the crystals are solubilized in the midgut and subsequently activated by proteases. The activated toxins bind to midgut epithelial cells and cause osmotic cell lysis, eventually leading to insect death.

Susceptibility of CPB to fungal entomopathogen *B. bassiana* has been intensively studied (Andrei, 1998).

Some *Fusarium* strains that don't cause damage to crop plants are also extensively studied for their possible use in biological control. Due to the highly specificity on insects, *Fusarium* spp. could cause high insect mortalities, especially of the Lepidoptera and Coleoptera orders. Some *Fusarium* species are weak, facultative pathogens and colonize their dead hosts as saprophytes. The entrance of the fungus is made via the oral route, oviposition tubes, wounds, or ectoparasitic activity. No penetration of the insect cuticle has been stated. Mycotoxins, such as trichothecenes (T-2) and other secondary metabolites, contributed to mortalities (Teetor-Barsch & Roberts, 1983).

Studies on bioassay of entomopathogenic microorganisms/bioproducts with insecticidal activity against CPB were carried out in Romania, at Research Development Institute for Plant Protection Bucharest (RDIPP) (Andrei, 1994, 1998, 2000, Andrei et al., 2001). The mass rearing of Colorado beetle is expensive and laborious so test insects could be a good option for pathogenicity test. The susceptibility to bioinsecticide (pathogenic mixture) of stored product pests that can be reared in laboratory all year round (Fatu et al., 2010; Dinu et al., 2013a, 2013b) and can provide a permanent source of entomological test material has been evaluated.

MATERIALS AND METHODS

Obtaining of bioinsecticide

Three native *B. bassiana* isolates (BbIt, BbAl and BbS1.07) were used in this experiment and they were selected on the basis of biological compatibility with selective pesticides. These isolates belong to RDIPP Bucharest entomopathogenic microorganisms collection and were deposited as BbIt NCAIM(P) F001392, BbAl NCAIM(P) F001386 and BbS1.07 NCAIM(P)F001353. Each *B.bassiana* isolate was cultivated separately on Goral medium (Goral, 1971). At the final period of incubation the concentration of fungal propagules were adjusted for each isolate at $2x10^9$cfu/ml. The *B. bassiana* fungal inoculum (V1) was obtained by mixing equal parts 1:1:1 (v/v/v) of fungal biomass.

The bacterial inoculum (V2) was obtained by cultivation of a commercial strain of *B. thuringiensis* on Nutrient broth. The final concentration of biomass was $3.2x10^3$cfu/ml.

An autochthonous strain of *F. oxysporum*, isolated from *L. decemlineata* larvae, was cultivated on PD broth resulting in a final concentration of 22.5×10^7conidia/ml representing the second fungal inoculum (V3).

Equal proportion (v/v/v) of *B. bassiana*, *B. thuringiensis* and *F. oxysporum* inoculum were mixed together in order to obtain the microbial complex inoculum (V4).

Bioassay of entomopathogenic mixture on *L. decemlineata* larvae

The test products, the pathogen mixture (V1), the pathogen mixture (V2 and V3), the pathogen (V4) mixture as well as the non-sterile distilled water (untreated control V5) and the chemical insecticide Nuprid chemical, (V6) were applied by foliar spraying.

Laboratory bioassay (Figure 1). The treatments were applied by sprinkling the potato dumplings, eggplants, to full coverage. For each variant, branches with 5 leaves were detached, in sterile Petri dishes, with filter paper. After spraying, the larvae were transferred on leaves and incubated for 14 days at 28°C. Within 10 days post-treatment, the plates were monitored daily for detection of dead larvae; the dead larvae in each treatment was recorded and counted. To confirm the fungal infection, any dead larva was placed on moistened filter paper and it was conducted macroscopic and microscopic morphological observation.

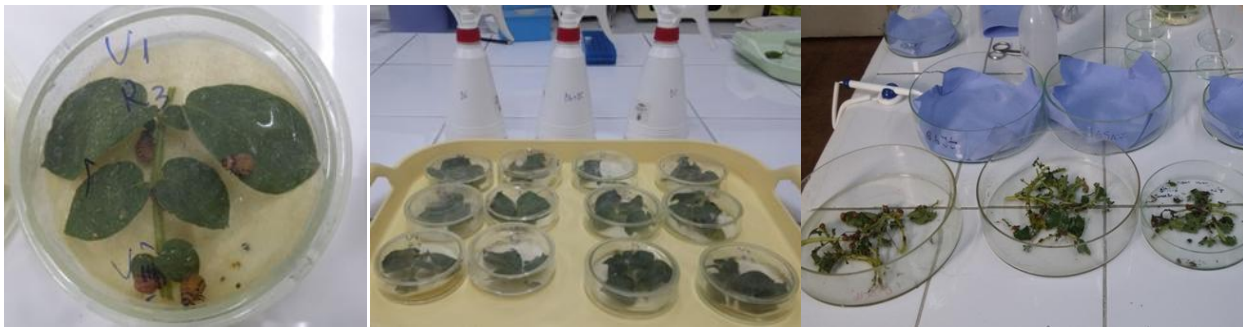

Figure 1. Laboratory bioassay on *L. decemlineata* larvae

Testing under isolator conditions was performed on potato plants covered with net baskets (Figure 2). The treatment was applied on leaves, corresponding to the dose of 450 l/ha.

Figure 2. Field bioassay on *L. decemlineata* larvae

Field bioassay on *L. decemlineata* larvae was performed in potato and eggplant cultures, by spraying the plants before flowering. Observations were made before treatment and 8 days after treatment, by recording live larvae. Efficacy was calculated using Haenderson-Tilton's formula (Henderson & Tilton, 1955).

Bioassay of entomopathogenic mixture on laboratory-reared insects: indian meal moth (*Plodia interpunctella*), greater wax moth (*Galleria mellonella*), mealworm beetle (*Tenebrio molitor*). The contamination of the culture medium was made by mixing fungal and bacterial biomass in Haydak's basic rearing medium. The larvae were placed in plastic boxes with perforated cover. Incubation was done in the thermostat chamber. The susceptibility assessment was based on the larval mortality rates recorded over 48 hours for a period of 10 days. The dead larvae in each treatment were recorded, counted and plotted.

RESULTS AND DISCUSSIONS

The confirmation of the larval death cause in experimental variants was made in "wet chambers" on the basis of specific symptoms of entomopathogenic microorganism's mode of action: white mycelium arising from the dead larvae confirmed fungal infection and dark color of the integument confirmed bacterial infection.

The contact component of the pathogenic mixture (V1) represented by *B. bassiana* conidial suspension ($2x10^9$ cfu / ml) applied at the dose of $9x10^{14}$cfu / ha had an efficacy of 90.93%, 8 days after application of the treatment (Figure 3).

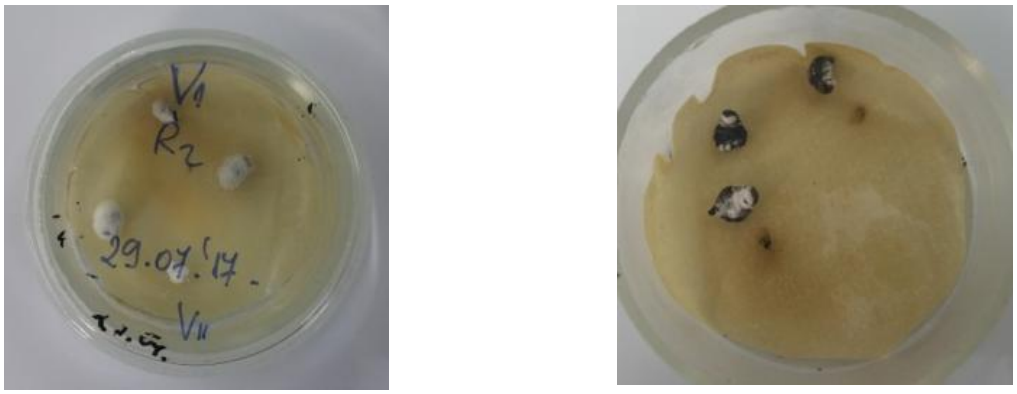

Figure 3. *B. bassiana* micosys on *L. decemlineata* larvae (V_1)

Figure 4. *F. oxysporum* micosys on *L. decemlineata* larvae (V_2)

The ingestion component of the pathogenic mixture (V2) represented by the *F. oxysporum* conidial suspension ($2.2x10^8$ cfu/ml) applied at the dose of $9.9x10^{13}$ cfu/ha had an efficacy of 6.23 %, 8 days after application of the treatment (Figure 4).

After *B. thuringiensis* treatment and the beginning of bacterial disease, we recorded some external symptoms on the infected larvae. It was observed interruption of feeding, paralysis and color changes. The integument of *L. decemlineata* larvae killed by bacteria

remained intact and it was dark in color - specific symptoms of bacteremia or septicemia resulted after the action of the bacterial toxins. The ingestion component of the pathogenic mixture (V3) represented by bacterial biomass and *B. th. tenebrionis* crystals ($3.2x10^3$cfu/ml) applied at the dose of $1.44x10^9$ cfu/ha had an efficacy of 92.28 %, 5 days after application of the treatment (Figure 5).

Figure 5. *B. th. tenebrionis* diseased larvae of *L. decemlineata* (V_3)

The pathogenic mixture (V4), represented by the suspension of fungal and bacterial spores, applied at the rate of 450 l/ha, reached an efficacy of 98.64%, 8 days after application of treatment (Figure 6).

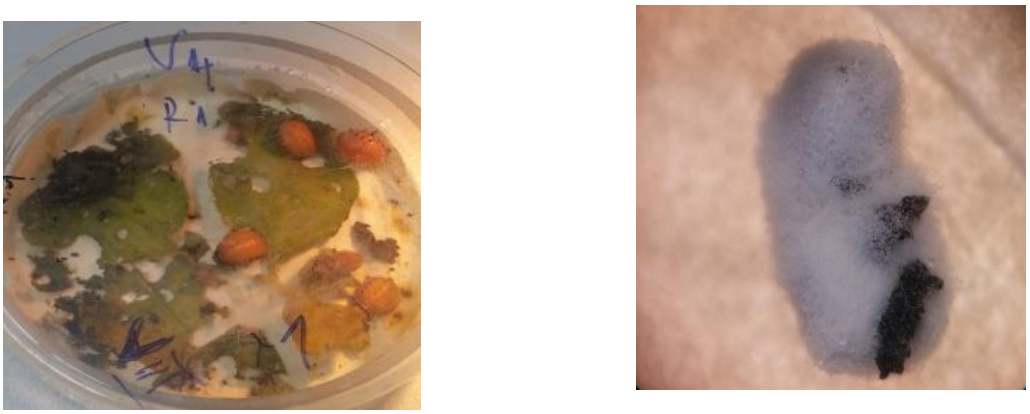

Figure 6. Diseased *L. decemlineata* larvae

In the untreated control variant (non-sterile water), no larval mortality was observed, the culture was completely compromised (Figure 7).

Figure 7. Healthy *L. decemlineata* larvae

Storage insects bioassay. In the bioassay diet procedure, mixing microbial biomass into the test insects feed substrate resulted in the test-doses presented in Table 1.

Table 1

Test-doses of the pathogenic inoculum in bioassay diet procedure

Variant (x 3 repetitions)	The titre of microbial suspensions	Pathogenic inoculum test dose / g medium	Test-dose of pathogenic inoculum / repetitions (10 larva)
(I) *B. bassiana*	2 x 10^9 cfu/ml	2.35 x 10^8	1.05 x 10^9
(II) *F. oxysporum*	2 x 10^8 cfu/ml	2.35 x 10^8	1.05 x 10^8
(III) *B. thuringiensis*	3.2 x 10^3 cfu/ml	3.76 x 10^2	1.69 x 10^3
(IV) Pathogenic mixture	I/II/III (v/v)		
(V) Pathogenic mixture	I/III (v/v)		

The results of the pathogen-induced larvae mortality test bioassays show different degrees of susceptibility of test insects to the pathogenic mixture (Figure 8, 9, 10, 11).

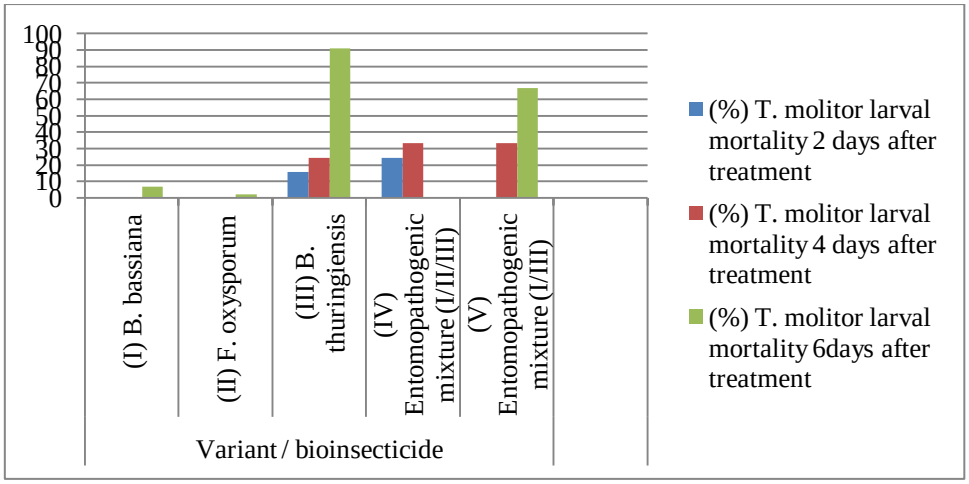

Figure 8. Evaluation of susceptibility of *T. molitor* test insect to pathogenic insecticide mixture

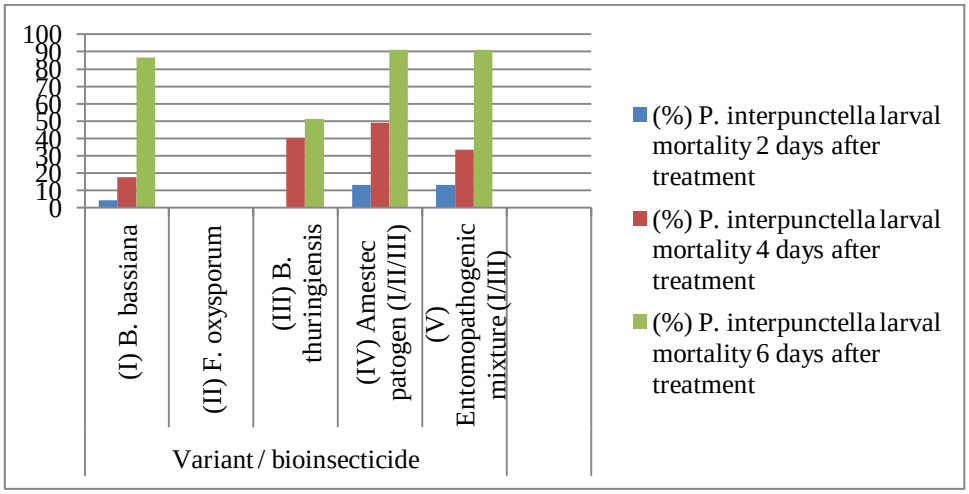

Figure 9. Evaluation of susceptibility of *P. interpunctella* test insect to pathogenic insecticide mixture

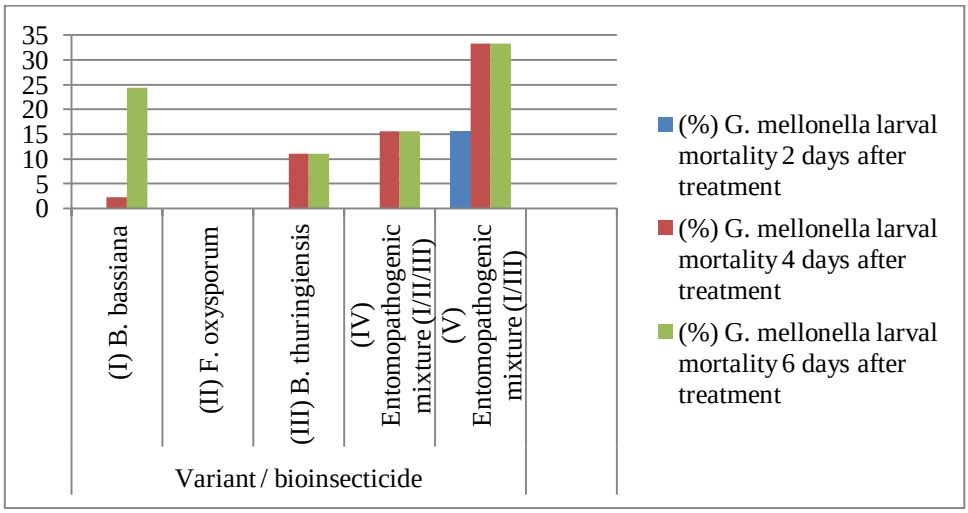

Figure 10. Evaluation of susceptibility of *G. mellonella* test insect to pathogenic insecticide mixture

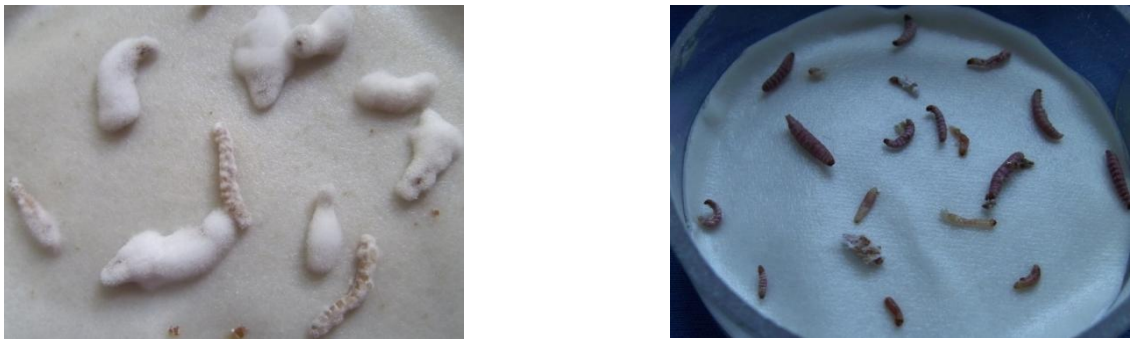

Figure 11. Storage test-insects recorded after entomopathogenic mixture application

P. interpunctella larvae showed the highest susceptibility to entomopathogenic mixture; when the mixture had two ingestion constituents (*B. thuringiensis* and *F. oxysporum*) and a contact constituent (*B. bassiana*), <10% larval mortality was recorded 48 hours after treatment, reaching > 90%, 6 days after treatment. Close mortality rates were also recorded when the entomopathogenic mixture was composed of one ingestion constituent (*B. thuringiensis*) and one contact constituent (*B. bassiana*), which proves that the *P. interpunctella* larvae are not susceptible to *F. oxysporum*, which corresponds to the lack of mortality in variant II at all observation intervals. Susceptibility of *T. molitor* larvae to *B. bassiana* and *B. thuringiensis* entomopathogenic mixture was low; larval mortality was recorded only 6 days after treatment (<70%). Low susceptibility to the entomopathogenic mixture (*B. thuringiensis* + *F. oxysporum*+ *B. bassiana*) was also revealed by *G. mellonella* larvae (15% mortality 6 days after treatment). The insecticidal effect of the entomopathogenic mixture increased by when the ingestion fungal component was eliminated (30% mortality, 6 days after application of the treatment). As in the case of *T. molitor* larvae, this is due to the lack of susceptibility of *G. mellonella* larvae to *F. oxysporum*.

The storage insects experimented in this study has been the subject of some papers about diets bioassay and the opinions of the specialists are different. Johnson et al. (1991) described a bioassay procedure for determining the level of toxicity of *B. thuringiensis* preparations toward the *P. interpunctella*, comparing results obtained in single larva bioassay technique with results from conventional long-term diet bioassay procedures. Portilla et al. (2014) described a non-autoclaved solid diet used to evaluate *B. bassiana* for control of the

tarnished plant bug, *Lygus lineolaris*. Regarding the potential of *T. molitor* as a bioassays insect-test, Bharadwaj and Stafford (2011) tested the yellow mealworm to indicate qualitatively the presence of entomopathogenic fungi in the soil or as a model for evaluating stress and other factors on fungal activity. Cotton and George (1929) consider that *T. molitor* are particularly problematic in bioassays because of the slower generation time.

Several different diets used in bioassay are developed. Johnson et al. (1991) used semi-dehydrated apple to screen the Indian meal moth for the effects of Bt toxins. Another bioassay with *T. molitor* larvae was developed by Herrero et al. (2001), using a flattened "pie-crust" of semi-hydrated cereal, wheat germ, yeast, and water, with sorbic acid and methylparaben, added to prevent mold. To evaluate the response of *T. molitor* larvae to insect control products, Oppert (2010) presented a simplified diet-disk assay method using punched disks of flattened whole-grain bread. Lestari and Rao (2016) used a dipping method to evaluate *Metarhizium* spp. and *Beauveria* spp. against *T. molitor* larvae. Association of entomopathogenic fungi to *T. molitor* have been studied to highlight the effect of different nutrients in fungal media in relation with (Savafi et al. 2007) and to observe immunity response against fungal pathogen infection (Moret et al., 2003). Zayed (2003) used the model insect *G. mellonella* reared on sterile bee wax, to evaluate the pathogenicity of some *B. bassiana* isolates according to their lethal potential. *G. mellonella* larvae are considered suitable in infection studies based on the injection of defined doses of bacteria. The most the most commonly and successfully insect used in bioassay of entomopathogens is *P. interpunctella*. Johnson et al. (1991) described a small scale bioassay procedure for determining the level of toxicity of *B. thuringiensis* preparations. Pathogenicity of entomopathogenic fungi to *P. interpunctella* measured by bioassay procedures are presented by Būda and Pečiulytė (2008).

CONCLUSIONS

The storage insects tested to develop a method to assess the biological quality of a complex bioinsecticide exhibited different degrees of susceptibility to the entomopathogenic mixture. *P. interpunctella* larvae showed the highest susceptibility. Infection of the larvae allowed monitoring bacterial and fungal virulence and examination of infection process. Also considering that *P. interpunctella* rearing is easy and relatively cheap, it was selected as test insect in a bioassay procedure of a complex bioinsecticide with ingestion and contact action against *L. decemlineata* larvae. The results of the presented pathogenicity studies are the basis of the optimized test procedure, which ensures the possibility of quality control of the pathogenic mixture (the bioassay) outside the vegetation period of the solanaceous crops.

ACKNOWLEDGEMENTS

This study was funded by ANCSI through the NUCLEU program PN 16-29-02-01 "*Sustainable pest management through the application of complex biological means and low-toxic chemicals*".

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